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Translation Efficiency of the Human T-Cell Leukemia Virus (HTLV-2) *gag* Gene Modulates the Frequency of Ribosomal Frameshifting

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The regulation of ribosomal frameshifting during translation of the polycistronic mRNA of human T-cell leukemia virus (HTLV) was studied in a cell-free system. Translation inhibitors such as cycloheximide and puromycin antibiotics were much more effective in blocking the synthesis of the frameshift polypeptide Gag–Pro than the synthesis of the Gag product. The preferential inhibition of the frameshift polypeptide Gag–Pro by the two antibiotics was not a reflection of the different sizes of the two gene products, but rather a consequence of the effect of the inhibitors on ribosomal translation efficiencies. To further analyze the effect of translation efficiencies on ribosomal frameshifting, we compared the translation of 5′-capped RNA to noncapped RNA. The translation of 5′-capped RNA was higher, as expected. Consequently, ribosomal frameshifting producing the Gag–Pro polypeptide was enhanced when compared to the translation of noncapped RNA. Taken together these results indicate that efficiencies of translation, in conjunction with the *cis* regulatory genetic elements at the frameshift sites, determine the ratio of the polypeptides Gag, Gag–Pro, and Gag–Pro–Pol produced in the HTLV-infected cell. Thus, physiological changes which affect the cellular translation machinery may alter the optimal ratio of these three polyprotein products needed for virus maturation. © 1995 Academic Press, Inc.

INTRODUCTION

The decoding of genetic information is an accurate process which ensures efficient and reliable transfer of the genetic information. Various biochemical mechanisms have evolved to minimize the rate of misreading of the genetic code. During translation elongation the maintenance of the translation frame is tightly controlled and random frameshifting is thus very low, 5×10^{-5} per codon (Kurland, 1992). Although very little is known about the mechanisms which maintain the frame reading, sequences in the mRNA which dictate programmed frame alteration during translation elongation are well characterized in both prokaryotic and eukaryotic systems (reviewed by Atkins *et al.*, 1990; Gesteland *et al.*, 1992).

Programmed frameshift sites usually consist of two elements, a recoding site and a stimulator. The change in frames occurs at the recoding site, which usually consists of a "slippery" heptanucleotide (Gesteland *et al.*, 1992). The slippery heptanucleotide is sufficient *in vitro* and *in vivo* to mediate a basal level of frameshifting (Wilson *et al.*, 1988; Reil *et al.*, 1993; Kollmus *et al.*, 1994). The stimulator usually consists of a RNA stem–loop secondary structure or pseudoknot 3′ adjacent to the recoding site (Jacks *et al.*, 1988; Brierley *et al.*, 1989; Atkins *et al.*, 1990). The RNA secondary structure is thought to stimulate the frameshifting event by causing a translational pause analogous to that produced by "hungry codons" during amino acid starvation in prokaryotic cells (Tu *et al.*, 1992).

Retroviruses employ two mechanisms of translational readthrough for the coordinated translation of the *gag* and the *pol* genes from the unspliced polycistronic viral mRNA: (a) suppression of the *gag* termination codon (Honigman *et al.*, 1991; Wills *et al.*, 1991; Hatfield *et al.*, 1992) and (b) ribosomal frameshifting (Jacks, 1990; Hatfield *et al.*, 1992; Atkins *et al.*, 1990). In human T-cell leukemia viruses (HTLV) two translational stop codons separate the *gag*, *pro*, and *pol* genes (Shimotohno *et al.*, 1985) and two events of −1 ribosomal frameshifts facilitate the synthesis of Gag–Pro and Gag–Pro–Pol polyproteins (Mador *et al.*, 1989; Falk *et al.*, 1993). The efficiency of frameshifting at the *gag–pro* junction is significantly higher than that at the *pro–pol* junction, as reflected by the ratio of the three polyproteins produced (Mador *et al.*, 1989). While the *cis*-acting signals which dictate ribosomal frameshifting of retroviral mRNAs have been well defined by genetic studies, there is a poor understanding about the biochemical mechanism of this unique translation event (see Farabaugh, 1993). No attempt has been made to evaluate the role of translation factors and ribosomes in the control of frameshifting.

It was previously demonstrated that the frequency of frameshifting at the HTLV distal *pro–pol* junction increased significantly following deletion or mutation of the proximal *gag–pro* termination signal (Mador *et al.*, 1989; Nam *et al.*, 1993). This deletion also increased the num-

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ber of ribosomes which reached the distal *pro–pol* junction. We also observed that the frequency of frameshifting increased when the *gag–pro* junction 41-nucleotide sequence was inserted into a heterologous mRNA (HIV-1 *nef*), in comparison to the frameshifting frequency between *gag* and *pro* in the HTLV-2 RNA. The *nef* RNA was shown before to be translated very efficiently in rabbit reticulocyte extracts (Kaminchik *et al.*, 1991; Falk *et al.*, 1993). Taken together, these results imply that the frameshift frequency may be affected by the efficiency of mRNA translation.

MATERIALS AND METHODS

Materials

Enzymes were purchased from Promega Corp. and radioactive materials from Amersham. Bacterial strain DR100 was used for plasmid manipulation and preparations (Laban and Cohen, 1981).

Plasmid construction

A 39-bp double-stranded synthetic DNA, which comprises the HTLV-2 *gag–pro* junction (nucleotides 2079–2117; Shimotohno *et al.*, 1985), and an additional two guanosine residues were inserted at the *Eco*RV restriction site (nucleotide 9152) of the HIV-1 *nef* gene in pNef (Kaminchick *et al.*, 1991). The addition of two guanosine residues at the 3′ end of the insert moved the ORF of the *nef* gene located distal (3′ end) to the insertion to a −1 position relative to the proximal part (5′ end) of *nef* (Fig. 1). The new clone was designated pNefHT. The sequence of the (+)-strand synthetic DNA 41-base insert was

5′ GAA AAA AAC TCC TTA AGG GGG GAG AT CTA ATC TCC CCC CGG 3′.
−1

Transcription and translation

The plasmid was linearized by cleavage with *Nde*I endonuclease downstream of the *nef* gene. The DNA was transcribed by SP6 RNA polymerase, and the RNA (2172 bases) was purified by phenol–chlorophorm extractions (Falk *et al.*, 1993). The NefHT RNA (1 μg) was translated in a reticulocyte extract (Promega Corp.) with [^{35}S]methionine (50 μCi/25 μl reaction mixture), as described before (Falk *et al.*, 1993). After 60 min of incubation at 30°, the radioactive polypeptide products were immunoprecipitated with rabbit polyclonal antibodies (5 μl) directed against the HIV-1 Nef protein (Kaminchik *et al.*, 1991). Total ^{35}S-labeled protein synthesis (G and GP) was estimated by measuring the radioactivity in the immunoprecipitate with a scintillation counter. Equal amounts (cpm) of the radioactive polypeptides were loaded for electrophoresis onto 15% SDS–polyacrylamide gels (SDS–PAGE), alongside molecular mass protein markers. The gels were dried down and [^{35}S]-methionine-labeled polypeptides (G and GP) were quantitated in a bioimaging analyzer (Fuji Corp.). Data obtained from the bioimaging analyzer are expressed as psl (photo-stimulated luminescence). The linearity of this measurement for [^{35}S]methionine was validated by comparison to that of scintillation radioactivity counting. At a range of 10–10,000 psl equivalent to 50–50,000 cpm, both measurements were linear.

RESULTS

Based upon the observations outlined in the Introduction (Mador *et al.*, 1989; Falk *et al.*, 1993), we hypothesized in the present study that the probability of a ribosome frameshifting once it encounters the proper *cis*-acting signal, is regulated by the efficiency of the mRNA translation. One of the major difficulties associated with such studies is the low frequency of ribosomal frameshifting (5%) dictated by most retroviral mRNAs, a level which is not amenable to accurate quantitation. To overcome this problem, we studied the HTLV-2 frameshift site within the context of the HIV-1 *nef* gene. Our previous studies with this fused RNA have demonstrated a very efficient translation as well as high levels of frameshifting, up to 40% (Falk *et al.*, 1993). The cloning of the HTLV-2 *gag–pro* frameshift sequence, 41 bases, was designed to introduce the UAA *gag* termination codon in frame with the 5′ end of the *nef* mRNA and at a −1 frame with respect to the 3′ end of *nef* mRNA (Fig. 1). Translation of this hybrid RNA (NefHT RNA) produces a Gag domain fused to the amino-terminus of Nef (designated G, 18 kDa) and a −1 frameshift hybrid polypeptide of the Gag–Pro junction flanked by Nef sequences at both the amino- and carboxy-termini (designated GP, 29 kDa). Since the *nef* mRNA has two independent AUG initiation sites, 60 nucleotides apart (Kaminchik *et al.*, 1991), two G and two GP polypeptides are actually translated (Fig. 1C). In order to accurately quantitate the two translation products (G and GP), polyclonal antibodies against Nef were used for immunoprecipitation and the hybrid polypeptides were subjected to electrophoresis on SDS–polyacrylamide gel (Fig. 2). Quantitation of the two ^{35}S-labeled translation products G and GP was facilitated by *in situ* analysis using a bioimaging analyzer.

In order to validate the capacity of the bioimaging analyzer to accurately measure levels of G and GP translation, we loaded onto the gel a series of twofold dilutions of the NefHT RNA translation reaction. Following electrophoresis the gel was dried and subjected to bioimaging analysis. As shown in Fig. 2A, the *in situ* radioactive analysis of G and GP is linear and the level of frameshifting is constant (24 ± 2%) irrespective of the absolute amount of radioactivity loaded on the gel. The same gel was also exposed to X-ray film and, as expected, linearity is difficult to observe due to overexposure of the film.

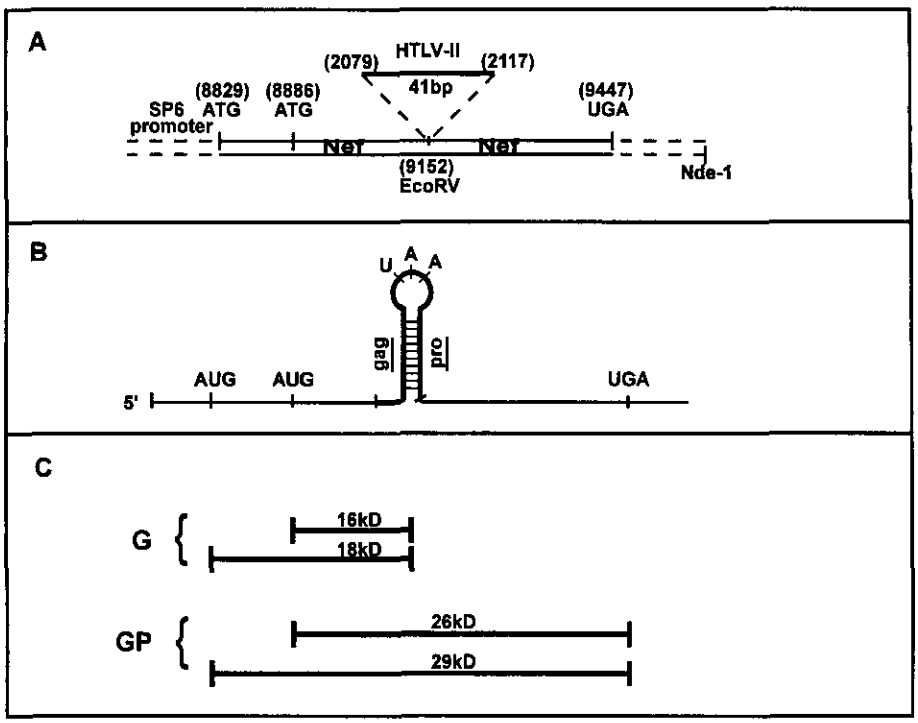

FIG. 1. Cloning of the HTLV-2 *gag–pro* junction into HIV-1 *nef* gene and production of the corresponding fused RNA and polypeptides. (A) A synthetic double-stranded DNA (41 bp) corresponding to the junction of HTLV-2 *gag–pro* (nucleotides 2079–2117 on the HTLV-2 sequence; Shimotohno *et al.*, 1985) was cloned into the *Eco*RV restriction site of HIV-1 *nef* (nucleotide 9152 in the HIV-1 sequence; Kaminchik *et al.*, 1991). The *nef* gene (nucleotides 8830–9610 in the HIV-1 sequence) was inserted into the polylinker of pSP6, giving rise to the final vector pNefHT (Falk *et al.*, 1993). (B) For preparation of RNA, the vector was linearized by *Nde*I endonuclease and transcribed with the SP6 RNA polymerase (Falk *et al.*, 1993). (C) The NefHT RNA (2172 bases) was purified and 1 μg RNA was translated in a nuclease-treated rabbit reticulocyte lysate. The expected polypeptide products are depicted (G and GP).

To investigate the possible correlation between efficiency of translation and ribosomal frameshifting levels, we first utilized translation inhibitors. The cycloheximide antibiotic (CHA) is known to interfere with translocation of ribosomes and thus to freeze polysome structures (Pestka, 1974). As expected, increasing concentrations of CHA gradually inhibited total protein synthesis in the reticulocyte extract (Fig. 3A). To specifically analyze the effect of CHA on the ratio of G to GP synthesis, a measure of frameshift frequency, equal amounts of immunoprecipitated [^{35}S]methionine-labeled polypeptides were used for gel electrophoresis. In the absence of CHA, the NefHT RNA directs synthesis of the expected products G and GP at a ratio of 83 to 17%, respectively. The inhibition of GP synthesis by CHA was stronger than that of G and therefore the ratio of the two translation products changed dramatically in a dose-dependent manner (Figs. 3B and 3C). These results suggest that ribosomal frameshifting was particularly sensitive to CHA, an inhibitor of ribosome translocation.

To further analyze the effect of translation inhibitors on the frameshift event, we selected the puromycin antibiotic. This antibiotic specifically interferes with ribosomal peptidyltransferase, to prematurely release the nascent polypeptide and thus reduce the average size of polysomes during protein synthesis (Pestka, 1974). Indeed, increasing concentrations of puromycin inhibited total protein synthesis in a dose-dependent manner (Fig. 4A). The synthesis of the frameshift polypeptide GP was again suppressed to a greater extent than that of the G polypeptide (Figs. 4B and 4C). In the presence of 2.5 μM puromycin, G synthesis was affected by less than twofold, while GP was suppressed by more than fivefold. At the high puromycin concentration of 10 μM, total protein synthesis was very low and therefore less [^{35}S]protein was loaded in lane 4 than in the other lanes. Nevertheless, since quantitation by the bioimaging analyzer of G and GP was linear in this range of radioactivity (Fig. 2), we included this reaction in Fig. 4.

The preferential inhibition of the frameshift product GP by the two antibiotics suggest that translation efficiency or ribosomal density along the mRNA affects frameshift frequencies. It could be argued, however, that the preferential inhibition of GP synthesis is a mere reflection of the target size and, in fact, synthesis of longer polypeptides would be more susceptible than short polypeptides to antibiotics. To test this possibility, we compared the inhibitory effect of CHA on the translation of Nef (27 kDa) to that of a truncated version of Nef (12 kDa) (Fig. 5). These two Nef products were chosen because they sim-

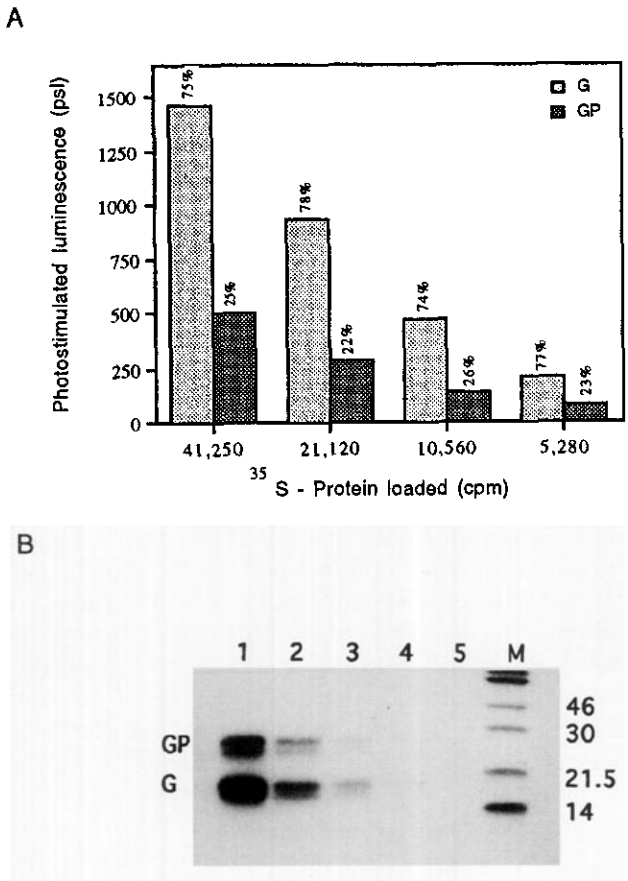

FIG. 2. Quantitation of G and GP translation products with a bioimaging analyzer. Following immunoprecipitation of the NefHT RNA translation products (Materials and Methods), the pellet was dissolved in 20 μl of SDS gel loading buffer. Twofold dilutions were performed with the same buffer and aliquots were taken for ^{35}S-labeled protein quantitation in a scintillation counter. Equal volumes of the protein dilutions (5280–41,250 cpm) were then subjected to gel electrophoresis and the dried gel was subjected to bioimaging analysis (A) and X-ray film exposure for 24 hr (B).

ulate the length of G and GP polypeptides shown in Figs. 3 and 4, but their synthesis does not involve frameshifting (see Fig. 1). Moreover, the methionine content of Nef and truncated Nef (four and two) is identical to that of GP and G polypeptides, respectively. Translation of the two RNAs, *Nde*I and *Eco*RV, under identical conditions gave yield to Nef (68,900 cpm) and truncated Nef (33,900 cpm), as expected (Fig. 5). The extent of inhibition by CHA is only slightly affected by the length of the translated polypeptide. Thus, the preferential inhibition of the frameshift GP polypeptide, shown in Figs. 3 and 4, indicates a specific effect on the frequency of frameshifting.

To further verify this conclusion, we sought a system in which translation efficiency could be modulated in the absence of inhibitors. It has been repeatedly shown that while noncapped synthetic mRNA can be accurately translated in a reticulocyte extract, translation efficiencies increased substantially when the same mRNA was 5′ capped (Melton *et al.*, 1984). We have thus prepared capped NefHT RNA by adding the cap analog structure $m^7G(5')PPP(5')G$ to the *in vitro* SP6 RNA polymerase transcription system. As was shown before, under proper

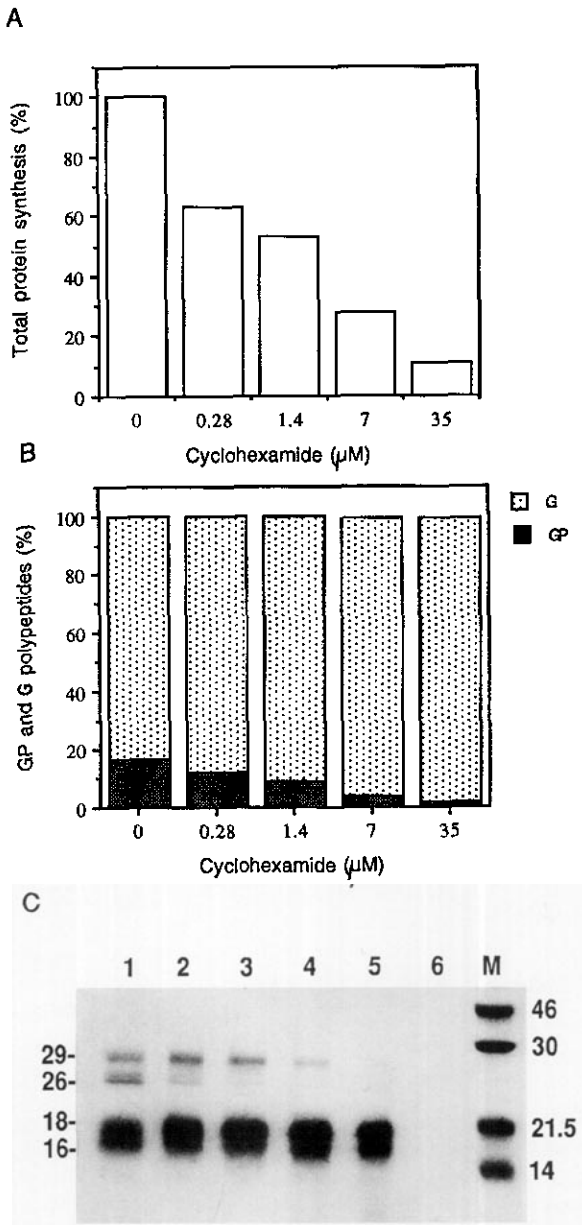

FIG. 3. Effect of cycloheximide on ribosomal frameshifting. The NefHT RNA was translated in the presence of increasing concentrations of cycloheximide (Sigma Corp.) and total radioactive protein synthesis was determined by scintillation counting following immunoprecipitation (A). Total protein synthesis without the inhibitor (100%) was 38,500 cpm. (B) Quantitation by bioimaging analyzer of the frameshift product GP (26 and 29 kDa) and the shorter product G (16 and 18 kDa) after SDS–PAGE of equal amounts of the radioactive immunoprecipitates. The psl values of GP and G polypeptides in a reaction without the inhibitor were 145 and 717 psl, respectively. The results are presented as the percentage of the GP and G products from the sum of the two radioactive products. (C) X-ray film exposure after SDS–PAGE of equal amounts of the immunoprecipitated radioactive products. Lanes 1–5, cycloheximide 0, 0.28, 1.4, 7, and 35 μM; lane 6, without RNA.

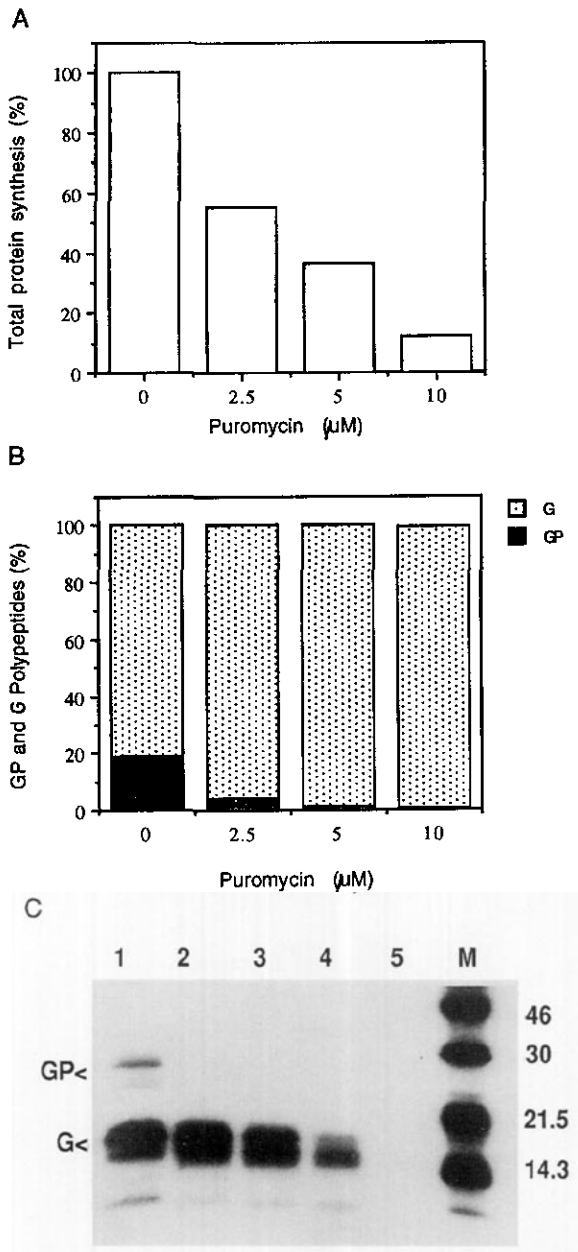

FIG. 4. Effect of puromycin on ribosomal frameshifting. The NefHT RNA was translated in the presence of increasing concentrations of puromycin (Sigma Corp.) and total protein synthesis was determined by a scintillation counter following immunoprecipitation (A). Total radioactive protein synthesis without the inhibitor (100%) was 35,450 cpm. (B) Quantitation of GP and G products after SDS–PAGE by bioimaging analyzer. The psl values of GP and G polypeptide without the inhibitor were 448 and 1925, respectively. (C) X-ray film exposure after SDS–PAGE of the immunoprecipitated radioactive products. Lanes 1–4, puromycin 0, 2.5, 5, and 10 μM; lane 5, without RNA. In lanes 1–3 equal amounts of ^{35}S-labeled proteins were loaded; at 10 μM concentration of puromycin (lane 4) total protein synthesis was very low and therefore less ^{35}S-labeled protein was loaded onto the SDS gel.

transcription conditions, at low GTP concentrations, synthesis of the mRNA was dependent on 5′ capping (Melton *et al.*, 1984). When increasing amounts of the 5′-capped and noncapped RNAs were translated under identical conditions, the capped RNA produced, as expected, higher (about twofold) amounts of [^{35}S]-methionine-labeled products. Analysis of the capped RNA translation products indicated an increase in frameshift frequency (20–25% GP polypeptide) relative to the noncapped RNA (10–12% GP polypeptide) (Figs. 6A and 6B). This result again indicates that frameshift efficiency may be modulated by the efficiency of translation.

DISCUSSION

Most of the studies on the mechanism of ribosomal frameshifting have concentrated on the genetic analysis of the RNA *cis*-acting signals. In this study, we demonstrated that the capacity of the *cis*-acting frameshift signals depends strongly upon the efficiency of mRNA translation. Enhancement (by the use of 5′-capped RNA) or decrease of the rate of translation (by the use of inhibitors) resulted in the modulation of frameshift frequencies.

Genetic signals that induce frameshifting usually include a "slippery" sequence followed by a secondary structure, stem and loop or a pseudoknot (Atkins *et al.*, 1991). It has also been demonstrated that ribosomes pause at the frameshift site, probably due to the secondary structure (Tu *et al.*, 1992). These observations are in general agreement with the hypothesis that ribosomes slip backward into a new reading frame when they encounter a specific RNA structure that affects their normal

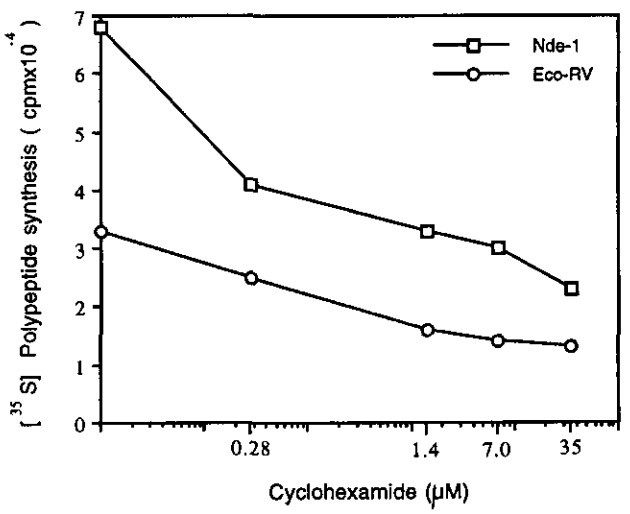

FIG. 5. Cycloheximide-mediated inhibition of Nef protein synthesis directed by long and short RNAs. Two RNAs were transcribed from the pNef vector(Kaminchik *et al.*, 1991) linearized either by *Eco*RV endonuclease or by *Nde*I endonuclease (see Fig. 1A). The two Nef RNAs (*Eco*RV and *Nde*I), transcribed from the same SP6 promoter, were 322 and 2131 bases, respectively. Translation of the two RNAs (1 μg) was carried out in parallel in the presence of increasing concentrations of cycloheximide. Total radioactive polypeptide synthesis was determined by scintillation counter following immunoprecipitation of the Nef products with anti-Nef rabbit sera. Protein synthesis without the inhibitor (100%) was 33,900 cpm for *Eco*RV RNA and 68,900 cpm for *Nde*I RNA.

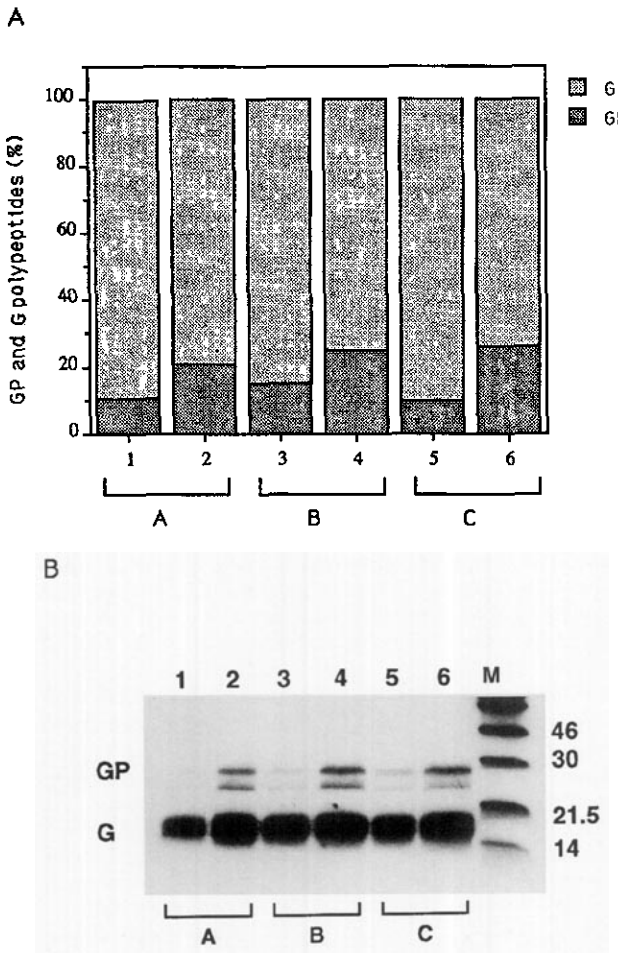

FIG. 6. Effect of 5′-end cap structure in mRNA on the efficiency of protein synthesis and ribosomal frameshifting. The 5′-end capped RNA was prepared in an SP6 transcription system in the presence of 0.5 m*M* $m^7G(5')ppp(5')G$ (Boehringer Corp.) as described by Melton *et al.*, 1984. The concentration of GTP was reduced to 0.05 m*M* and ATP, GTP, and CTP were kept at the standard concentration (0.5 m*M*). Under these conditions NefHT RNA synthesis was dependent upon the addition of the cap structure GpppG (Melton *et al.*, 1984). This was also demonstrated by analysis of the RNA products by electrophoresis on agarose gels (1%) and staining with ethidium bromide (data not shown). (A) Increasing amounts of uncapped and 5′-end capped NefHT RNAs were translated and total protein synthesis was determined following immunoprecipitation. Lanes 1 and 2, 1 μg of uncapped and 5′-end capped RNAs, respectively. Lanes 3 and 4, 2 μg of uncapped and 5′-end capped RNAs, respective. Lanes 5 and 6, 4 μg of uncapped and 5′-end capped RNAs, respectively. (B) Quantitation of the GP and G translation products after SDS–PAGE of equal amounts of the radioactive immunoprecipitates by bioimaging analyzer. The psl values of GP and G polypeptides were 76 and 653 psl, respectively, for 1 μg uncapped RNA and 386 and 1443 psl for 1 μg capped RNA (lanes A); 196 and 1110 psl for 2 μg uncapped RNA and 455 and 1382 psl for 2 μg capped RNA (lanes B); 129 and 1190 psl for 4 μg uncapped RNA and 404 and 1129 psl for 4 μg capped RNA (lanes C).

progression along the mRNA (Jacks *et al.*, 1988). Two contradictory events are taking place near the frameshift site, translation termination at the stop codon on one hand and a −1 reframing on the other hand. The relative efficiency of each of these processes should affect the ratio of the Gag and Gag–Pro products. Usually the ribosome pauses at the stop codon to facilitate binding of the release factor for translation termination. This event is further modulated by additional factors such as the secondary structure of the mRNA and the termination codon sequence context. The major rate-limiting factors that determine the kinetics of translation termination are the release factors (Wolin and Walter, 1988; Tate and Brown, 1992). In theory, a delay in termination while waiting for the release factor may enhance the opportunity for the alternative event of frameshifting. However, we have previously shown that mutation of the HTLV *gag* termination codon UAA to a sense codon does not affect the frequency of frameshifting to produce the Gag–Pro polyprotein (Falk *et al.*, 1993). This could indicate that the frameshifting event is independent of translation termination. It does not preclude the possibility that termination of the Gag is affected by the pausing of ribosomes at the frameshift signal of HTLV mRNA.

How does translation efficiency affect the frequency of frameshifting? The methods that we have used to modulate translation have all affected polysome sizes, and therefore perhaps frameshift frequencies. (i) Cycloheximide, which blocks translocation, reduces average polysome size (Jimenez *et al.*, 1977); (ii) puromycin induces premature release of nascent polypeptide and thus the dissociation of the corresponding 80S ribosomes from the polysome (Pestka, 1974); and (iii) the rate of initiation enhanced by the 5′-cap structure increases polysome size. We propose, therefore, that in addition to ribosome pausing at both the frameshift site and the termination codon, the distance in between ribosomes near the frameshift site determines the frequency of reframing and thus the ratio of Gag to Gag–Pro. Taken together, these findings suggest that infectious virus assembly is dependent on the capacity of the cell to support efficient translation of the retroviral polycystronic mRNA to produce the proper ratio of Gag to the frameshift polypeptides.

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